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Stereochemistry of a bifunctional dihydroceramide Δ^4-desaturase/hydroxylase from *Candida albicans;* a key enzyme of sphingolipid metabolism

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The stereochemical course of the dihydroceramide Δ^4-(*E*)-desaturase from *Candida albicans*, cloned and expressed in the yeast *Saccharomyces cerevisiae* strain *sur2*Δ, was determined using stereospecifically labelled (2*R*,3*S*)-[2,3,4,4-$^2H_4$]-palmitic acid as a metabolic probe. Mass spectrometric analysis of the dinitrophenyl-derivatives of the labelled long-chain bases revealed elimination of a single deuterium atom from C(4) (corresponding to the C(4)-H_R) along with a hydrogen atom from C(5) (corresponding to the C(5)-H_S). This finding is consistent with an overall *syn*-elimination of the two vicinal hydrogen atoms. Besides the desaturation product sphingosine (93%) minor amounts of a 4-hydroxylated product (phytosphinganine, 7%) were identified that classify the *Candida* enzyme as a bifunctional desaturase/hydroxylase. Both processes, desaturation and hydroxylation proceed with loss of C(4)-H_R from the chiral precursor. This finding is in agreement with a two-step process involving activation of the substrate by removal of the C(4)-H_R to give a C-centred radical or radicaloid followed by either disproportionation into an olefin, water and a reduced diiron complex, or to recombination of the primary reactive intermediate with an active site-bound oxygen to yield a secondary alcohol. This result demonstrates the close mechanistic relationship between desaturation and hydroxylation as two different reaction pathways of a single enzyme and strengthens the mechanistic relationship of desaturases from fatty acid metabolism and sphingolipids.

Introduction

Sphingolipids comprise a large class of highly bioactive compounds with diverse biological functions. A central compound within sphingolipid metabolism is ceramide, which serves as a precursor of all other major sphingolipids.[1,2] Ceramide is known to be involved in many important biological processes such as control of metabolism *via* ceramide-activated protein-kinases and -phosphatases, cell cycle arrest, cell differentiation and apoptosis. Ceramide can be either biosynthesised *de novo* or upon breakdown of more complex sphingolipids.[3,4] The *de novo* biosynthesis of ceramide starts with a serine palmitoyl-transferase which catalyses the condensation of palmitoyl-CoA and serine to form 3-ketosphinganine which is further processed to sphinganine.

Subsequently, sphinganine is *N*-acylated by transfer of an acyl-residue to give dihydroceramides. The final step of this *de novo* ceramide biosynthesis involves a dihydroceramide Δ^4-(*E*)-desaturase which converts the dihydroceramides into ceramides (Scheme 1). This reaction is of great importance, since it transforms the less active dihydroceramides into highly active ceramides.[5] From a mechanistic point of view, sphingolipid desaturases seem to be closely related to the much better investigated fatty acid desaturases. Two major groups of fatty acid desaturases have been characterised; a large family of membrane bound desaturases and a smaller group of soluble desaturases, the latter only found in plants. The membrane-bound enzymes have a multi-histidine diiron coordination site and catalyse in a stereo- and regioselective manner an oxygen-dependent *syn*-dehydrogenation of non-activated aliphatic/olefinic substrates linked to CoA- or glycerolipids. The process of desaturation is initiated by an enzyme-bound iron-oxo species abstracting a single and specific hydrogen atom from the substrate. This energetically demanding homolytic cleavage of a non-activated C–H bond is associated with a large kinetic isotope effect (KIE; k_H/k_D approx. 5–8) and is assumed to generate a carbon centred radical (Scheme 2) for all membrane-bound enzymes.[6,7]

Then, a second hydrogen atom from a neighbouring methylene group is transferred to the diiron-centre deactivating the highly reactive intermediate(s) by disproportionation into an olefin, water and a reduced iron-complex. The loss of the second hydrogen atom is not associated with a noticeable KIE. In addition, some desaturases exhibit an intrinsic residual hydroxylase activity directed against the same methylene group where desaturation starts, most likely due to a common reactive intermediate that is channelled either into desaturation or hydroxylation.[8]

First data on the stereochemical course of a dihydroceramide Δ^4-(*E*)-desaturase became available in the early seventies. *In vivo* studies with preparations from rat brain and tritium labelled precursors gave rise to a kinetic isotope effect (KIE) for the abstraction of a *pro-R* hydrogen atom from C(4) of a sphinganine precursor along with a second hydrogen atom from C(5) consistent with an overall *syn*-elimination.[9] Other authors arrived at an overall *anti*-elimination.[10] Since the previous results were based on experiments with tissue preparations lacking the strength of approaches with cloned genes and heterologously expressed enzymes and considering the contradictory reports on the stereochemistry of the desaturation, we were encouraged to re-address this question using transformed yeast cultures overexpressing a dihydroceramide Δ^4-(*E*)-desaturase which is lacking in wild type cells. A recent bioinformatics approach afforded a family of protein sequences from animals,

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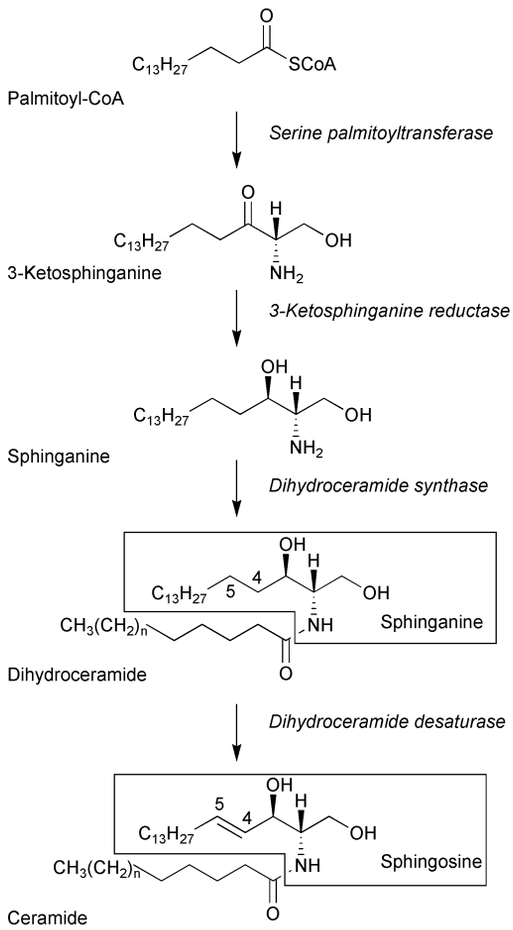
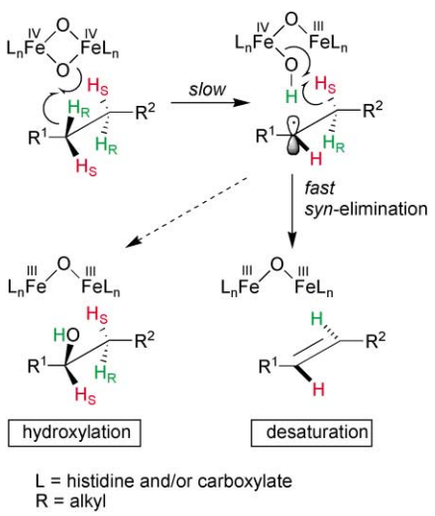


Scheme 1 *De novo* biosynthesis of ceramide.



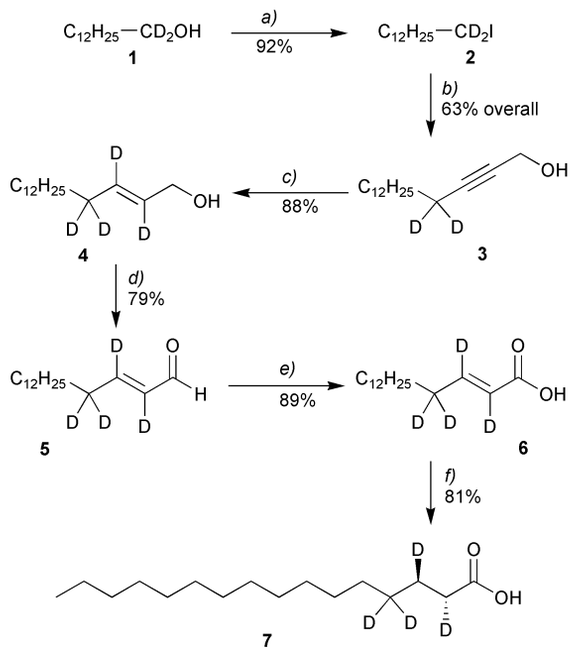
Scheme 2 General mechanism for desaturation and hydroxylation of aliphatic substrates (Ref. 6). The oxidising species is assumed to be an electrophilic 1,2-bis-μ-oxo complex of Fe(IV) or an diiron-(III,IV)-oxo species after bridge-to-terminal oxo migration (not shown,[29]). Both processes involve removal of the same hydrogen atom from the substrate as the initial step.

plants and fungi which were subsequently cloned and expressed in a *Saccharomyces cerevisiae* mutant strain (sur2Δ) lacking long-chain base C4-hydroxylation.[11] Biochemical characterisation of the transgenic yeast revealed that these proteins belong to the family of sphingolipid Δ^4-(*E*)-desaturases (dihydroceramide desaturases). Here, we report on a novel stereochemical analysis of the desaturation step using the heterologously expressed dihydroceramide Δ^4-(*E*)-desaturase from *Candida albicans* as a model system. We also demonstrate that this enzyme has a dual catalytic function, namely desaturation and hydroxylation of dihydroceramide.

Results and discussion

Synthesis of (2*R*,3*S*)-[2,3,4,4–$^2H_4$]palmitic acid

As a model for the stereochemical analysis of a dihydroceramide Δ^4-(*E*)-desaturase, the corresponding enzyme from *Candida albicans* was cloned and expressed in the *Saccharomyces cerevisiae* strain *sur2*Δ.[11] The stereochemistry of the desaturation process was investigated with stereospecifically labelled precursors. After inhibition of the fatty acid biosynthesis using cerulenin,[12] externally added palmitic acid is taken up by the yeast cells, converted into acyl-CoA and channelled into the sphingolipid metabolism along the pathway outlined in Scheme 1. Owing to the efficiency of this biocatalytic conversion of palmitic acid into dihydroceramide, the synthetic approach can focus on the synthesis of chiral [2,3,4,4-$^2H_4$]-(2*R*,3*S*)-palmitic acid **7**. After incorporation, this acid is converted into the labelled dihydroceramide **8** with two deuterium atoms residing at the carbon atoms that are directly involved in the desaturation process and two others at C(6) facilitating an unambiguous identification of the metabolite in case of a simultaneous removal of the deuterium atoms from C(4) and C(5) (Fig. 1). The isotope labels are easily introduced by the synthetic protocol outlined in Scheme 3.



Scheme 3 Synthesis of (2*R*,3*S*)-[2,3,4,4-$^2H_4$]-palmitic acid. a) I_2, imidazole, PPh_3; b) 1. Li–C≡C–CH_2–O–THP, 2. *p*-toluenesulfonic acid–MeOH; c) $LiAl^2H_4$, ^2HCl, ^2H_2O; d) MnO_2; e) $NaClO_2$; f) anaerobic biocatalytic H_2-reduction with broken cells of *Clostridium tyrobutyricum* (strain C. La1; for the stereochemical analysis see Experimental section).

Reduction of methyl tridecanoate with lithium aluminium deuteride (98% ^2H) afforded [1,1-$^2H_2$]-tridecan-1-ol **1**. Alcohol **1** was converted into the corresponding iodide **2** and alkylated with the lithium salt of 2-prop-2-ynyloxytetrahydropyran in liquid ammonia. After removal of the protecting group, the alkynol **3** was reduced with lithium aluminium deuteride followed by hydrolysis of the organoaluminium intermediates with ^2H_2O and ^2HCl. Oxidation of the alkenol **4** to the corresponding acid **6** was achieved in two steps by treatment of **4** with manganese dioxide and subsequent oxidation of the resulting aldehyde **5** with sodium chlorite in a buffered aqueous solution. Configurationally pure [2,3,4,4-$^2H_4$]-hexadec-2-enoic acid

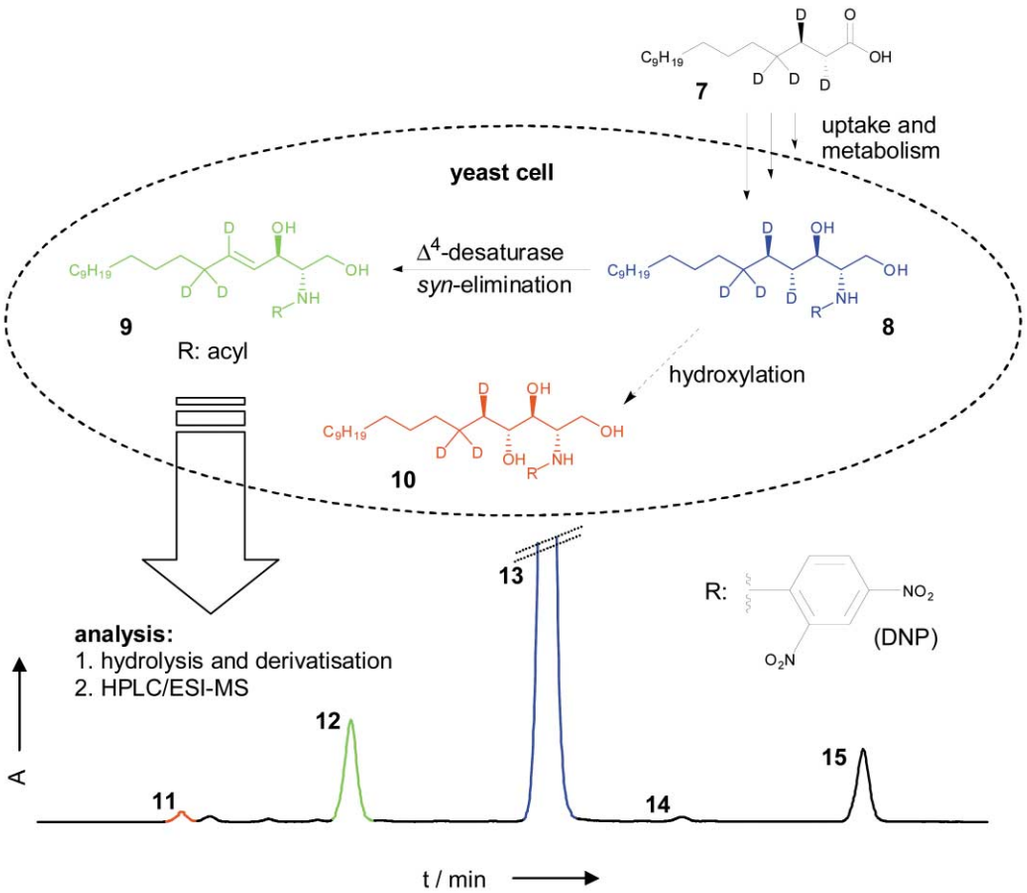


Fig. 1 Strategy of feeding experiments and analysis of long-chain bases. Metabolites were released by hydrolysis, extracted and converted into their DNP-derivatives. After TLC purification, separation and identification of the DNP-long-chain bases was achieved by HPLC and mass spectrometry: **11**, C_{18}-4-hydroxysphinganine; **12**, C_{18}-sphingosine; **13**, C_{18}-sphinganine; **14**, C_{20}-sphingosine and **15**, C_{20}-4-hydroxysphinganine.

6 was thus obtained in 70% overall yield. The key step of the asymmetric synthesis is the biocatalytic reduction of the labelled acid **6** with broken cells of *Clostridium tyrobutyricum* (strain C. La1) and hydrogen gas.[13,14] The reduction of the α,β-unsaturated acid proceeds stereospecifically with an *anti*-2*Si*,3*Si*-transfer of two hydrogen atoms across the double bond of the precursor and afforded (2*R*,3*S*)-[2,3,4,4-$^2H_4$]-palmitic acid **7** in 81% yield and very high enantiomeric excess (>97% per centre) as shown by ^1H NMR after conversion of **7** into the corresponding mandelate diester.[15]

Stereochemical analysis of the dihydroceramide Δ^4-(*E*)-desaturase

*Sur2*Δ cells of *Saccharomyces cerevisiae,* lacking the genuine C(4)-hydroxylase gene (due to disruption of the *SUR2* gene)[16] but overexpressing the dihydroceramide desaturase from *Candida albicans,*[11] were grown in the presence of deuterium-labelled palmitic acid **7** and cerulenin. This fungal metabolite strongly inhibits the yeast's intrinsic ability to synthesise fatty acids[12] and, hence, minimises the competition between externally added precursors and genuine fatty acids in the serine palmitoyl transferase reaction. To increase the internal substrate level of dihydroceramide, the transgenic cells were subjected to heat shock.[17,18] Following conversion of the labelled palmitic acid **7** into dihydroceramide **8**, the sphingolipid was desaturated to labelled ceramide **9** (R = acyl) by the heterologously expressed *Candida* enzyme. For analysis of the whole spectrum of products, the sphingolipids of whole cells were hydrolysed, and the liberated sphingobases converted with *Sanger's* reagent into their dinitrophenyl (DNP) derivatives as described before.[19] The DNP derivatives were pre-purified by TLC (SiO_2 60) and finally separated and identified by HPLC (RP18) and mass spectrometry using electrospray ionisation (ESI-MS) in the negative ion mode (Fig. 1). Besides labelled DNP-sphinganine **13** (85%) and its C_{20} homolog **15** (6%), two unsaturated DNP-sphingenines **12** (7%) and **14** (1%), resulting from desaturation of **8** and its C_{20} homologue, and a DNP-trihydroxysphinganine **11** (<1%) were identified.

All DNP-derivatives of sphingoid metabolites displayed intense $[M - H]^-$ ions. The efficiency of the conversion of **7** into labelled dihydroceramide **8** is demonstrated by Fig. 2B showing the molecular species of labelled DNP-sphinganine [$^2H_4$]-**13** (*m*/*z* = 470, 100%) and of native unlabelled [^1H]-**13** (*m*/*z* = 466, 52%) corresponding to approx. 60% labelled material. Owing to a large kinetic isotope effect during desaturation[20] the ratio of labelled DNP-sphingosine [$^2H_3$]-**12** (*m*/*z* = 467) to native [^1H]-**12** (*m*/*z* = 464) drops to approx. 1 : 0.95, Fig. 2C. The molecular species of [$^2H_3$]-**12** is consistent with a clear and uniform loss of a single deuterium atom together with a single hydrogen atom. Since the desaturation process involves only hydrogen atoms from C(4) and C(5) of dihydroceramide [$^2H_4$]-**8**, two of the remaining deuterium atoms have to reside on C(6), the other one either on C(4) or on C(5) of the labelled DNP-sphingosine **12**.

The C_{20} homologue is also desaturated and yields tetradeuterated DNP-dihomosphingosine **14** (*m*/*z* = 496) with complete retention of all deuterium atoms originally present in the precursor. This is in agreement with a two-carbon chain elongation of the administered [$^2H_4$]-palmitic acid **7**.

Interestingly, the cluster of the pseudomolecular ions of the early eluting DNP-derivative of labelled C_{18}-trihydroxysphinganine **11** (see Fig. 1), at *m*/*z* = 485/486, demands for a predominant, but not exclusive loss of a deuterium atom ([$^2H_3$]-**11**: [$^2H_4$]-**11**, approx. 1 : 0.6) from C(4) of dihydroceramide **8** (corresponding to the C(4)-H_R, Fig. 2A).[9,10,21] Control

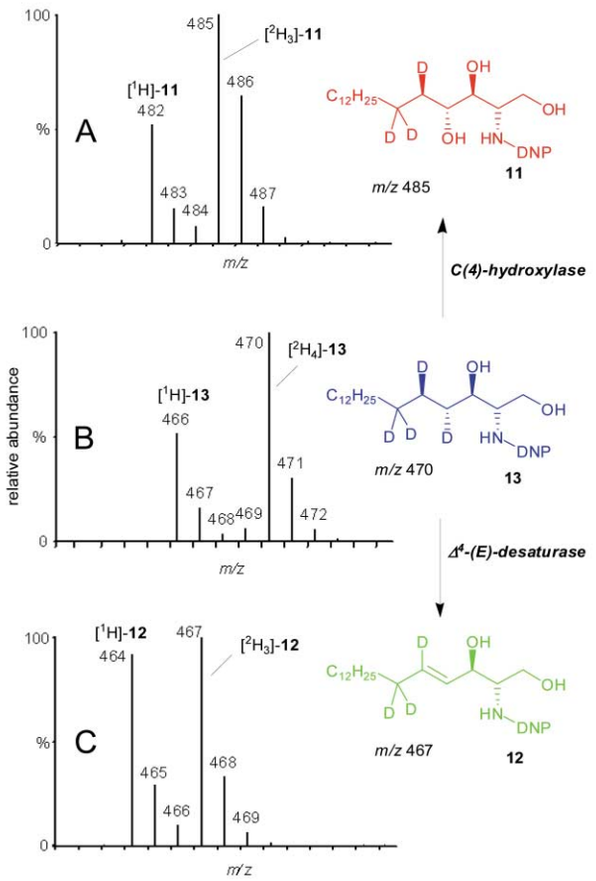
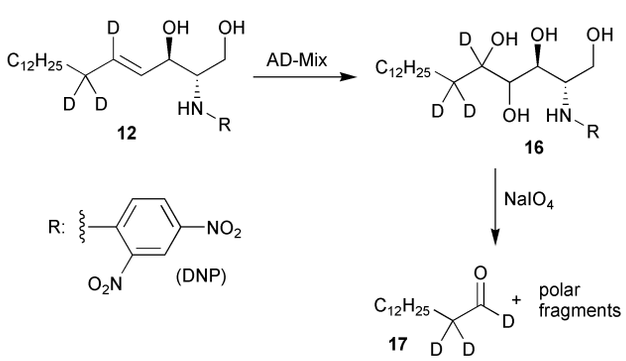


Fig. 2 Mass spectrometric analysis of deuterium-labelled metabolites. Shown are the clusters of pseudomolecular ions from DNP-4-hydroxysphinganine **11** (Fig. 2A), DNP-sphinganine **13** (Fig. 2B), and DNP-sphingosine **12** (Fig. 2C). The group of pseudomolecular ions at higher masses (*e.g.* m/z = 485, 486, and 487, Fig. 2A) corresponds to the labelled metabolites, the cluster at lower masses (*e.g.* m/z = 482, 483, and 484, Fig. 2A) is due to unlabelled, native compounds.

experiments with yeast cells expressing the empty vector of pYES2 do not produce any desaturation products or hydroxylated sphinganines and, hence, the labelled DNP-derivative of [$^2H_3$]-4-hydroxysphinganine **11** has to be likewise considered as a product of the expressed *Candida* desaturase. Moreover, treatment of the extract (see Fig. 1) with excess of $NaIO_4$ resulted in a quantitative cleavage of **11** and, thus, independently confirmed the structure of this metabolite as a vicinal 3,4-dihydroxy compound. All other compounds shown in Fig. 1 were not affected by the reagent.

In order to determine the position of the remaining deuterium atom at the double bond of deuterium-labelled ceramide **9**, the corresponding DNP fraction was collected by HPLC and subjected to an oxidative degradation yielding tetradecanal **17** from the aliphatic terminus of the metabolite **12** (Scheme 4). The cleavage was achieved following a recently published



Scheme 4 Oxidative degradation of DNP-sphingosine **12**.

protocol[22] involving dihydroxylation of the (4*E*)-double bond[23] to give **16** followed by cleavage of the carbon backbone with sodium periodate under phase transfer conditions.[24] Since aliphatic aldehydes generally display very weak molecular ions under EI-MS conditions, the resulting tetradecanal was analysed using chemical ionisation with isobutane. [1,2,2-$^2H_3$]-Tetradecanal **17** from oxidative degradation of **12** displayed the presence of three deuterium atoms accompanied by minor amounts of di- and monodeuterated isotopomers and unlabelled aldehyde ($^2H_3$–$^2H_2$–$^2H_1$ approx. 100 : 35 : 10). Prolonged reaction times (24 h) in ^1H_2O further decreased the deuterium content, whereas running the reaction in ^2H_2O had the opposite effect. Accordingly, a slow exchange of the acidic α-hydrogens of the aldehyde is responsible for the variable isotopomer composition and not the desaturation process.

In summary, the stereochemical course of the desaturation by the *Candida* enzyme can be unambiguously described as a *syn*-elinination of the C(4)-H_R along with the C(5)-H_S hydrogen atom from the dihydroceramide precursor **8** as outlined in Scheme 2. Most importantly, with respect to the C(4)-H_R both catalytic activities of the enzyme, namely hydroxylation and desaturation, start by removal of the same hydrogen atom from the carbon backbone of the ceramide precursor.

Conclusions

The total number of known and suspected non-heme diiron enzymes is steadily increasing because of the wealth of sequence data becoming available from genome-sequencing and bioinformatics efforts.[25] Recently, this resulted in the identification of a family of dihydroceramide Δ^4-(*E*)-desaturases from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster* and *Candida albicans* along with a bifunctional Δ^4-desaturase/hydroxylase from *M. musculus*.[11] From these examples the heterologously expressed enzyme from *C. albicans* was chosen as a model system for a detailed mechanistic and stereochemical analysis of the desaturation process. In agreement with all other previously examined cases, the enzyme operates stereo- and regiospecifically with simultaneous *syn*-elimination of two vicinal hydrogen atoms, namely the C(4)-H_R and the C(5)-H_S. This stereochemical course is fully consistent with the results obtained by Stoffel *et al.*[9] Buist *et al.* studied the intermolecular kinetic isotope effects of a dihydroceramide Δ^4-(*E*)-desaturase from rat liver microsomes and found that the introduction of the double bond occurs in two discrete steps with a significant KIE (k_H/k_D = 8.0 ± 0.8) for the abstraction of the C(4)-H atom and almost no KIE for the loss of the hydrogen atom from C(5) (k_H/k_D = 1.02 ± 0.07).[20] In agreement with other fatty acid desaturases, the KIE is generally observed for the carbon atom proximal to the polar head of the substrate molecule. A notable exception for the class of membrane-bound enzymes is the recently investigated non-specific Δ^8-(*E,Z*)-sphingolipid desaturase which obeys the rule in case of its *trans*-product (attack onto C(8)-H_R), but switches to the distal carbon (attack onto the C(9)-H_R) in the case of its *cis*-product.[22] In contrast, the soluble stearoyl-ACP Δ^9-desaturase from castor seed showed no significant KIE for either the C(9) or C(10) (k_H/k_D approx. 1) probably due to masking by another, kinetically more important step in the catalytic cycle.[26]

Apparently, the Δ^4-desaturase from *C. albicans* is also able to act as a C(4) hydroxylase as minor amounts of a corresponding C_{18} trihydroxysphinganine **10** were identified among the reaction products (Fig. 1). Since the hydroxylation, like the desaturation process, removes the same C(4)-H_R, both processes apparently generate the same reactive intermediate, namely a C(4)-centred radical or radicaloid that is channelled predominantly (approx. 93%) into desaturation or recombines with an active-site bound oxygen atom to give an alcohol (approx. 7%, formal insertion of oxygen into the C(4)-H_R bond). The factors controlling this ratio remain to be established in detail, but

might be largely due to a proper positioning of the substrate at the active site. First information on this aspect came from the exchange of four amino acids in core positions of an oleate desaturase of *Arabidopsis thaliana* which converted the enzyme into a bifunctional desaturase/hydroxylase generating different product ratios.[8] The importance of substrate presentation at the active site is also obvious from studies with the mechanistically related methane monooxygenase which converts less tightly bound artificial substrates such as ethyl benzene into an alcohol and an olefin.[27] Both findings, namely modification of the active site as well as the conversion of non-natural substrates resulting in different ratios of desaturation *versus* hydroxylation, are consistent with a uniform mechanistic view for both pathways concerning the early steps. In case of desaturation, activation of the substrate generates a reactive intermediate (C-centred radical) whose orientation and conformation at the active site only allows a disproportionation into an olefin, water and a reduced diiron complex. The hydroxylation probably requires a slightly different orientation of the substrate relative to the diiron-centre. After formation of the same primary radical as before, the divergent substrate orientation allows a recombination with the iron-bound hydroxy group yielding an alcohol along with the reduced diiron-centre (Scheme 1). Whether the two conformationally different enzyme–substrate complexes represent the entry points into the respective catalytical cycles or emerge during the progress of the reaction is currently unknown and remains to be established. Investigations into this direction are under way with the bifunctional Δ^4-(*E*)-desaturase/hydroxylase from mouse and will be reported in due course.

Experimental

General methods

Reactions were performed under Ar. Solvents were dried according to standard methods or commercially available dry solvents (Fluka) were used instead. IR: Bruker Equinox 55 FTIR Spectrophotometer. ^{1}H and ^{13}C NMR: Bruker AV 400 (Bruker, D-76287 Rheinstetten/Karlsruhe, Germany). Chemical shifts of ^{1}H and ^{13}C NMR are given in ppm (δ) based on solvent signals: $CDCl_3$ 7.26 ppm (^{1}H NMR) and 77.16 ppm (^{13}C NMR). For ESI-LC-MS measurements a Micromass Quattro II (Micromass, Manchester, UK) connected to an Agilent HP1100 HPLC, equipped with a RP18-column (125 mm × 2 mm, 3 µm, Grom D-71083 Herrenberg, Germany) was used. Gas chromatography/mass spectrometry (70 eV) was performed on a Micromass MasSpec (Micromass, Manchester, UK) double-focusing magnetic sector field mass spectrometer (geometry EBE) connected to a Hewlett Packard HP6890 II gas chromatograph, equipped with a DB-5 (J&W Scientific) nonpolar capillary column (30 m × 0.25 mm, 0.25 µm). Silica gel: Si 60 (0.200–0.063 mm, E. Merck, Darmstadt, Germany) was used for chromatography.

Expression system and incubation experiments

The plasmid pYES2 (Invitrogen) containing the open reading frame of the Δ^4-(*E*)-sphingolipid desaturase from *Candida albicans* was used to transform the strain *sur2*Δ of *Saccharomyces cerevisiae*.[11] Transgenic sur2Δ cells were grown in complete minimal medium (110 ml) dropout uracil[28] containing 2% (w/v) raffinose as a carbon source, 2% (w/v) galactose to induce expression, 25 µM cerulenin (Sigma) and 250 µM labelled palmitic acid **12**. Cultures were grown aerobically at 25 °C for 6 d (final OD_{600} ~0.8), subjected to a heat shock at 37 °C for 90 min, and harvested by centrifugation. Cells (~ 300 mg fresh weight) were washed and directly hydrolysed (10% $Ba(OH)_2$ (w/v) in H_2O–dioxane, 1 : 1, 24 h, 110 °C). The released long chain sphingobases (LCB) were converted into the dinitrophenyl derivatives (DNP-derivatives) and pre-purified by thin layer chromatography (silica gel 60, $CHCl_3$–MeOH 9 : 1, v/v) as described before.[19]

[1,1-$^2H_2$]-Tridecan-1-ol (1)

A solution of methyl tridecanoate (10 g, 44 mmol) in abs. THF (20 ml) was added slowly within 10 min at rt to a suspension of lithium aluminium deuteride (1.0 g, 24 mmol, 98% ^{2}H) in the same solvent (50 ml). After 1 h at reflux, the mixture was cooled to 0 °C and hydrolysed with water (10 ml) and dil. HCl (6 M). Extractive work-up with ether, drying and removal of solvent under reduced pressure gave alcohol **1** as a colourless solid. Yield: 8.4 g (95%). Mp: 32.5–33.5 °C. δ_H (400 MHz, $CDCl_3$) 0.87 (3 H, t, *J* 7.1, CH_3), 1.22–1.36 (20 H, m, 10 × CH_2), 1.38 (1 H, br s, OH), 1.54 (2 H, t, *J* 7.2, $CH_2C^2H_2$); δ_C(100.6 MHz, $CDCl_3$) 14.24, 22.83, 25.84, 29.49, 29.59, 29.74, 29.76, 29.79, 29.80, 29.83, 32.06, 32.76, 62.46 (qui., *J* 21.5, C^2H_2); *m*/*z* (EI) 184.21588 ($M^{+\bullet}$ − H_20, 32%. $C_{13}H_{24}{}^2H_2$ requires 184.21601) 156 (14), 112 (16), 97 (44), 84 (74), 70 (93), 57 (100); ν_{max}(neat)/cm^{-1} 3305br, 2959, 2923, 2852, 1463, 1375, 1166, 1130, 1104, 1073, 962, 713.

[1,1-$^2H_2$]-1-Iodotridecane (2)

A chilled solution of triphenylphosphine (13.11 g, 50 mmol) and imidazole (3.40 g, 50 mmol) in diethyl ether–acetonitrile (200 ml, v/v, 3 : 1) was treated with iodine (12.69 g, 50 mmol) in four portions within 20 min. The suspension was allowed to come to rt, chilled and slowly treated with alcohol **1** (7.4 g, 37 mmol). Stirring was continued for 1 h at rt, followed by addition of light petroleum (200 ml). The upper layer was decanted, the residual phase treated with aq. $NaHCO_3$ (5%, 150 ml) and extracted with light petroleum (3 × 50 ml). The organic layers were concentrated under reduced pressure and the residue dissolved in pentane (2 × 50 ml). After drying, evaporation of solvent *in vacuo* (i.v.), the iodide was purified by flash chromatography on silica gel using light petroleum for elution. Colourless oil. Yield: 10.5 g (92%). δ_H(400 MHz, $CDCl_3$) 0.90 (3 H, t, *J* 6.8, CH_3), 1.21–1.33 (18 H, m, 9 × CH_2), 1.38 (2 H, qui., *J* 7.1, C(3)H), 1.83 (2 H, t, *J* = 7.4, $CH_2C^2H_2$); δ_C(100.6 MHz, $CDCl_3$) 7.08 (qui., *J* 22.9, C^2H_2), 14.25, 22.83, 28.71, 29.50, 29.57, 29.70, 29.77, 29.79, 29.81, 30.61, 32.07, 32,51; *m*/*z* (EI) 312.12946 ($M^{+\bullet}$, 14%. $C_{13}H_{25}{}^2H_2I$ requires 312.12831), 185 (45), 87 (28), 85 (32), 73 (34), 71 (56), 59 (40), 57 (100); ν_{max}(film)/cm^{-1} 2955, 2930, 2853, 1466, 1377, 1124, 1107, 978, 887, 721.

[4,4–$^2H_2$]-Hexadec-2-yn-1-ol (3)

Finely cut lithium wire (0.35 g, 50 mmol) was added slowly to a well stirred solution of liquid ammonia (250 ml) and iron(III) nitrate nonahydrate (10 mg, 0.025 mmol) at −78 °C. After complete conversion into the amide the solution turned gray and 2-prop-2-ynyloxytetrahydropyran (7.00 g, 50 mmol) was added over a period of 10 min. Stirring was continued for 1 h at −60 °C, and pre-cooled dry DMSO (100 ml) was added followed by slow addition of the iodide **2** (10 g, 32 mmol) within 15 min. The mixture was allowed to warm to rt overnight and the ammonia evaporated. The residue was dissolved in water (250 ml) and extracted with diethyl ether (3 × 200 ml). The organic extracts were washed with water (50 ml), dried and concentrated under reduced pressure. Chromatography on silica gel using light petroleum–diethyl ether (v/v, 9 : 1) for elution gave the THP ether of **3** as a colourless oil. Yield: 7.0 g (67%). A solution of the THP ether (6.5 g, 20 mmol) and *p*-toluenesulfonic acid hydrate (200 mg, 1 mmol) in methanol (50 ml) was stirred for 1 h at rt. Pyridine (1 ml) was added prior to evaporation of solvents under reduced pressure. The residue was treated with water (25 ml) and extracted with diethyl ether (3 × 50 ml). The organic extracts were dried and concentrated under reduced pressure. Column chromatography on silica gel

using light petroleum–diethyl ether (v/v 4 : 1) for elution afforded alcohol **3** as a colourless solid. Yield: 4.5 g (94%). Mp: 54–55 °C. δ_H(400 MHz, $CDCl_3$) 0.87 (3 H, t, *J* 6.9, CH_3), 1.21–1.40 (20 H, m, 10 × CH_2), 1.45–1.51 (2 H, m, $CH_2C^2H_2$), 1.63 (2 H, br s, OH), 4.24 (2 H, s, CH_2OH); δ_C(100.6 MHz, $CDCl_3$) 14.24, 18.30 (qui., *J* 18.4, C^2H_2), 22.83, 28.57, 28.97, 29.30, 29.50, 29.66, 29.77, 29.79, 29.80, 29.83, 32.06, 51.56, 78.44, 86.77; *m*/*z* (EI) 222.23164 ($M^{+\bullet}$ − H_20, 4%. $C_{16}H_{26}{}^2H_2$ requires 222.23166), 137 (33), 123 (45), 113 (67), 95 (77), 83 (100), 69 (91); ν_{max}(neat)/cm^{-1} 3250br, 2954, 2914, 2852, 1472, 1259, 1091, 1042, 1024, 1006, 798.

[2,3,4,4-$^2H_4$]-(2*E*)-Hexadec-2-en-1-ol (4)

A solution of alcohol **3** (4.5 g, 19 mmol) in dry THF (20 ml) was added in 5 min to a suspension of lithium aluminium deuteride (0.95 g, 22.5 mmol) in dry THF (50 ml). The mixture was refluxed for 2 h and cooled to 0 °C followed by hydrolysis with ^2H_2O (2.0 ml) and cautious addition of ^2HCl (7.5 ml, 36% in ^2H_2O). The resulting semi-solid mixture was then acidified by dilute hydrochloric acid (1 M) until a milky suspension was obtained. The aq. layer was extracted with diethyl ether (3 × 50 ml), and the organic extracts washed with sat. $NaHCO_3$ solution (20 ml) and water (20 ml). After drying and evaporation of solvents the alkenol was obtained as a colourless solid. Yield: 4.1 g (88%). Mp: 36.5–37.5 °C. δ_H(400 MHz, $CDCl_3$) 0.88 (3 H, t, *J* 7.0, CH_3), 1.21–1.38 (22 H, m, 11 × CH_2), 4.08 (2 H, s, CH_2OH); δ_C(100.6 MHz, $CDCl_3$) 14.2, 22.84, 29.11, 29.29, 29.51, 29.66, 29.76, 29.81, 29.82, 29.83, 29.84, 31.44 (qui., *J* 19.4, C^2H_2), 32.08, 63.91, 128.59 (t, *J* 23.8, C^2H), 133.25 (t, *J* 23.1, C^2H); *m*/*z* (EI) 244.27045 ($M^{+\bullet}$, 4%. $C_{16}H_{28}{}^2H_4O$ requires 244.27042), 226 (14), 141 (8), 127 (13), 99 (45), 85 (63), 71 (55), 59 (100); ν_{max}(KBr)/cm^{-1} 3298br, 2917, 2849, 1473, 1043, 1009, 713.

[2,3,4,4-$^2H_4$]-(2*E*)-Hexadec-2-en-1-al (5)

A solution of the alkenol **4** (3.67 g, 15 mmol) in CH_2Cl_2 (50 ml) was treated while stirring at rt with activated manganese(IV) oxide (10 g, 115 mmol). After vigorous shaking for 1 h another portion of manganese dioxide (5 g, 58 mmol) was added and shaking continued for 1 h. The oxidant was allowed to settle, and the supernatant was decanted. The decantation was repeated (3 × 50 ml dichloromethane), and the combined extracts were filtered over a pad of Celite. After drying, and removal of solvent *in vacuo*, chromatography on silica gel using light petroleum–diethyl ether (v/v, 4 : 1) for elution gave aldehyde **5** as a colourless liquid. Yield: 2.87 g (79%). δ_H(400 MHz, $CDCl_3$) 0.90 (3 H, t, *J* 7.1, CH_3), 1.21–1.40 (20 H, m, 10 × CH_2), 1.51 (2 H, br t, *J* 7.0, C(5)H), 9.50 (1 H, s, CHO); δ_C(100.6 MHz, $CDCl_3$) 14.22, 22.81, 27.81, 29.21, 29.47, 29.48, 29.63, 29.73, 29.77 (2 × CH_2), 29.79, 31.96 (qui., *J* 19.4, C^2H_2), 32.04, 132.81 (t, *J* 24.6, C(2)), 158.49 (t, *J* 22.7, C(3)), 194.17 (CHO); *m*/*z* (EI) 242.25461 ($M^{+\bullet}$, 25%. $C_{16}H_{26}{}^2H_4O$ requires 242.25477), 224 (16), 139 (22), 125 (36), 97 (51), 83 (67), 74 (100), 57 (92); ν_{max}(KBr)/cm^{-1} 2955, 2913, 2845, 1692, 1665, 1601, 1468, 1395, 1171, 723.

[2,3,4,4-$^2H_4$]-(2*E*)-Hexadec-2-enoic acid (6)

A solution of sodium chlorite (6.2 g, 55 mmol) and sodium dihydrogenphosphate (8.0 g, 67 mmol) in water (50 ml) was added over 20 min to aldehyde **5** (1.81 g, 7.5 mmol) in *tert*-butanol (70 ml) and pent-1-ene (15 ml). The yellow opalescent solution was stirred for 2 h followed by evaporation of solvent *in vacuo*. Water (50 ml) was added and the aqueous phase extracted with diethyl ether (3 × 75 ml). The combined extracts were washed with aq. NaOH (3 × 75 ml, 1 M solution), acidified (pH 2–3) with 6 M hydrochloric acid and extracted with diethyl ether (3 × 75 ml). After drying and removal of solvents the acid **6** was obtained as a colourless solid. Yield: 1.73 g (89%). Mp: 46.5–48 °C. δ_H(400 MHz, $CDCl_3$) 0.88 (3 H, t, *J* 6.9, CH_3), 1.19–1.36 (20 H, m, 10 × CH_2), 1.45 (2 H, m, CH_2), 11.84 (1 H, br s, COOH); δ_C(100.6 MHz, $CDCl_3$) 14.24, 22.84, 27.86, 29.24, 29.51 29.53, 29.67, 29.77, 29.80, 29.81, 29.83, 31.58 (qui., *J* 18.5, C^2H_2), 32.08, 120.48 (t, *J* 24.6, C(2)), 152.14 (t, *J* 23.1, C(3)), 172,48 (COOH); *m*/*z* (EI) 258.24952 ($M^{+\bullet}$, 13%. $C_{16}H_{26}{}^2H_4O_2$ requires 258.24969), 240 (39), 197 (12), 130 (18), 116 (27), 102 (41), 98 (49), 84 (49), 70 (58), 57 (100); ν_{max}(KBr)/cm^{-1} 2963, 2923, 2848, 1662, 2555, 1681, 1468, 1419, 1290, 1059, 931, 789, 718.

(2*R*,3*S*)-[2,3,4,4-$^2H_4$]-Hexadecanoic acid (7)

The sodium salt of **6** (1 g, 3,9 mmol) was dissolved in potassium phosphate buffer (50 ml, 0.1 M, pH = 7) and placed in a reaction vessel with a thermostat. Methyl viologene (15 mg, 0.06 mmol) and tetracycline (3.0 mg) were added and the solvent was degassed by passing a gentle stream of helium for 1 h through the solution. The system was flushed with argon and approx. 5 g broken cells of *Clostridium tyrobutyricum* (strain C. La1, DSM number 1460) were added with strict exclusion of oxygen. Then, argon was replaced by hydrogen (connected to gas burette to monitor the progress of the reaction) while the vessel was gently shaken at 35 °C. After a brief induction period the suspension turned deep blue. After 48 h no further consumption of hydrogen was observed and the reaction mixture was acidified with dil. H_2SO_4 (10%) to pH 2–3. The mixture was continuously extracted with diethyl ether for 12 h. The ether layer was dried and concentrated under reduced pressure. Chromatography on silica gel with light petroleum–diethyl ether (v/v, 1 : 2) yielded the chiral palmitic acid **7** as a colourless solid. Yield: 0.75 g (81%). Mp: 61.5–62.5 °C. For incubation experiments the acid was additionally purified by semi-preparative HPLC (RP 18) using a linear gradient from methanol–water (90 : 10) to 100% methanol within 20 min. δ_H(400 MHz, $CDCl_3$) 0.88 (3 H, t, *J* 7.1, CH_3), 1.21–1.37 (22 H, m, 12 × CH_2), 1.59 (1 H, d, *J* 8.4, CH_2), 2.32 (1 H, d, *J* 8.4, CH_2), 11.25 (1H, br s, COOH); δ_C(100.6 MHz, $CDCl_3$) 14.27, 22.85, 24.21 (t, *J* 19.4, C(3)), 28.26 (qui., *J* 19.3, C^2H_2), 29.17, 29.52, 29.54, 29.76, 29.80, 29.81, 29.83, 29.84, 29.85, 32.09, 33.82 (t, *J* 19.4, C(2)), 180.57 (COOH); *m*/*z* (EI) 260.26551 ($M^{+\bullet}$, 100%. $C_{16}H_{28}{}^2H_4O_2$ requires 260.26534), 214 (14), 133 (30), 119 (10), 74 (41), 57 (42); ν_{max}(KBr)/cm^{-1} 2964, 2954, 2916, 2848, 1698, 1471, 1353, 1312, 1296, 1257, 1221, 940, 675.

Analysis of the enantiomeric purity of (2*R*,3*S*)-[2,3,4,4–$^2H_4$]-hexadecanoic acid (7)

(2*R*,3*S*)-[2,3,4,4-$^2H_4$]-Palmitic **7** acid was converted with methyl (*S*)-(+)-mandelate into the corresponding mandelate diester.[15,30] The ^1H NMR spectrum was recorded with simultaneous irradiation at 1.6 ppm to suppress the vicinal coupling of the protons at C(3) and C(2). The signal of the proton C(2)-H_R (>98%) of the labeled acid appears as a singlet at 2.20 ppm while the minor C(2)-H_S is observed at 2.24 ppm (<2%). Within the error limits of the NMR method the enantiomeric excess of the labeled palmitic acid **7** is ≥96%.

Isolation and analysis of long-chain bases

The DNP-spingoid bases were separated by HPLC under reversed phase conditions (GROM-SIL 120 ODS-5 ST, 3 μm, 125 × 2 mm, Grom, Herrenberg) with a flow rate of 0.2 ml min^{-1}. A linear gradient from 60% MeOH–CH_3CN–propan-2-ol (v/v/v 10 : 3 : 1) and 40% water to 20% water within 10 min and finally to 0% water within further 40 min was used. The ESI-MS spectra were recorded in the negative ion mode (source temperature: 100 °C, desolvation temperature: 250 °C, cone voltage: 35 Volt).

Oxidative cleavage of DNP-sphingosine

The DNP-derivative of [5,6,6-$^2H_3$]-sphingosine **12** was collected by HPLC and concentrated under reduced pressure to give approx. 1 μg of product. AD-mix-α (14.0 mg, Aldrich), *t*-BuOH–water (100 μl, v/v, 1 : 1) and a catalytic amount of methane sulfonamide was added. The resulting two phase system was vigorously shaken for 12 h at rt. A sat. aq. solution of sodium sulfite (100 μl) was added and shaking was continued for 5 min. After extraction with ethyl acetate (3 × 200 μl) the combined solvents were removed with a gentle stream of argon. The residue was dissolved in 100 μl dichloromethane, and a catalytic amount of benzyltrimethylammonium chloride and sodium periodate (20 μl, sat. aq. solution) was added. The mixture was shaken for 5 min and aliquots were directly analysed by GC/MS using chemical ionisation with isobutane.

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References

1 Y. A. Hannun and L. M. Obeid, *J. Biol. Chem.*, 2002, **277**, 25847–25850.
2 T. Kolter and K. Sandhoff, *Angew. Chem., Int. Ed.*, 1999, **38**, 1532–1568.
3 Y. A. Hannun, C. Luberto and K. M. Argraves, *Biochemistry*, 2001, **40**, 4893–4903.
4 A. Huwiler, T. Kolter, J. Pfeilschifter and K. Sandhoff, *Biochim. Biophys. Acta Mol. Cell. Biol. Lipids*, 2000, **1485**, 63–99.
5 A. H. Merrill, Jr., *J. Biol. Chem.*, 2002, **277**, 25843–25846.
6 B. Behrouzian and P. H. Buist, *Curr. Opin. Chem. Biol.*, 2002, **6**, 577–582.
7 A. Svatos, B. Kalinova and W. Boland, *Insect Biochem. Mol. Biol.*, 1999, **29**, 225–232.
8 J. A. Broadwater, E. Whittle and J. Shanklin, *J. Biol. Chem.*, 2002, **277**, 15613–15620.
9 W. Stoffel, G. Assmann and K. Bister, *Hoppe-Seyler's Z. Physiol. Chem.*, 1971, **352**, 1531–1544.
10 A. J. Polito and C. C. Sweeley, *J. Biol. Chem.*, 1971, **246**, 4178–4187.
11 P. Ternes, S. Franke, U. Zähringer, P. Sperling and E. Heinz, *J. Biol. Chem.*, 2002, **277**, 25512–25518.
12 F. Schneider, R. Lessire, J. J. Bessoule, H. Juguelin and C. Cassagne, *Biochim. Biophys. Acta*, 1993, **1152**, 243–252.
13 H. Simon, J. Bader, H. Gunther, S. Neumann and J. Thanos, *Angew. Chem. Int. Ed. Engl.*, 1985, **24**, 539–553.
14 O. Thum, C. Hertweck, H. Simon and W. Boland, *Synthesis*, 1999, 2145–2150.
15 G. Görgen, W. Boland, U. Preiss and H. Simon, *Helv. Chim. Acta*, 1989, **72**, 917–928.
16 D. Haak, K. Gable, T. Beeler and T. Dunn, *J. Biol. Chem.*, 1997, **272**, 29704–29710.
17 G. M. Jenkins, A. Richards, T. Wahl, C. G. Mao, L. Obeid and Y. Hannun, *J. Biol. Chem.*, 1997, **272**, 32566–32572.
18 R. C. Dickson, E. E. Nagiec, M. Skrzypek, P. Tillman, G. B. Wells and R. L. Lester, *J. Biol. Chem.*, 1997, **272**, 30196–30200.
19 P. Sperling, U. Zähringer and E. Heinz, *J. Biol. Chem.*, 1998, **273**, 28590–28596.
20 C. K. Savile, G. Fabrias and P. H. Buist, *J. Am. Chem. Soc.*, 2001, **123**, 4382–4385.
21 W. Stoffel and E. Binczek, *Hoppe-Seyler's Z. Physiol. Chem.*, 1971, **352**, 1065–1072.
22 C. Beckmann, J. Rattke, N. J. Oldham, P. Sperling, E. Heinz and W. Boland, *Angew. Chem., Int. Ed.*, 2002, **41**, 2298–2300.
23 H. C. Kolb, M. S. Vannieuwenhze and K. B. Sharpless, *Chem. Rev.*, 1994, **94**, 2483–2547.
24 T. Takata, R. Tajima and W. Ando, *J. Org. Chem.*, 1983, **48**, 4764–4766.
25 P. Sperling, P. Ternes, T. K. Zank and E. Heinz, *Prostaglandins, Leukotrienes Essent. Fatty Acids*, 2003, **68**, 73–95.
26 B. Behrouzian, P. H. Buist and J. Shanklin, *Chem. Commun.*, 2001, 401–402.
27 Y. Jin and J. D. Lipscomb, *J. Biol. Inorg. Chem.*, 2001, **6**, 717–725.
28 D. A. Treco and V. Lundblad, in *Current Protocols in Molecular Biology*, eds. F. M. Asubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl, L. M. Albright, D. M. Coen, and A. Varki, John Wiley & Sons, 1993.
29 L. Que and W. B. Tolman, *Angew. Chem., Int. Ed.*, 2002, **41**, 1114–1137.
30 D. Parker, *J. Chem. Soc., Perkin Trans. 2*, 1983, 83–88.